

TRANSLATOR'S DECLARATION

I, Janet Hope, BSc(Hons.), MIL., MITI., translator to Messrs. Taylor and Meyer of 20 Kingsmead Road, London, SW2 3JD, Great Britain, verify that I know well both the German and the English language, that I have prepared the attached English translation of 44 pages of a German Patent application in the German language with the title:

Verfahren zur fermentativen Herstellung von D-Pantothensäure unter Verwendung coryneformer Bakterien

identified by the code number 000439 BT / IP at the upper left of each page and that the attached English translation of this document is a true and correct translation of the document attached thereto to the best of my knowledge and belief.

I further declare that all statements made of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements are made with the knowledge that wilful false statements and the like are punishable by fine or imprisonment, or both, under 18 USC 1001, and that such false statements may jeopardize the validity of this document.

Signed:

A handwritten signature in cursive script, appearing to read "J Hope".

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The attached papers are a true and accurate reproduction of the original documents for this patent application.

Munich, 29th August 2001

**On behalf of the President of the German
Patent and Trade Mark Office**

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Brand

Process for the fermentative preparation of D-pantothenic acid using coryneform bacteria

The invention provides a process for the fermentative preparation of D-pantothenic acid using coryneform bacteria
5 in which the poxB gene is attenuated.

Prior art

Pantothenic acid is a vitamin of commercial importance which is used in human medicine and in the pharmaceuticals industry, in the foodstuffs industry and very particularly
10 in animal nutrition.

Pantothenic acid can be prepared by chemical synthesis, or biotechnologically by fermentation of suitable microorganisms in suitable nutrient solutions. In the chemical synthesis, DL-pantolactone is an important
15 intermediate stage. It is prepared in a multi-stage process from formaldehyde, isobutylaldehyde and cyanide. In further process steps, the racemic mixture is separated, D-pantolactone is subjected to a condensation reaction with β -alanine, and the desired D-pantothenic acid is obtained
20 in this way.

The advantage of the fermentative preparation by microorganisms lies in the direct formation of the desired stereoisomeric D-form, which is free from L-pantothenic acid.

25 Various types of bacteria, such as e.g. Escherichia coli (E. coli), Arthrobacter ureafaciens, Corynebacterium erythrogenes, Brevibacterium ammoniagenes, and also yeasts, such as e.g. Debaromyces castellii, can produce D-pantothenic acid in a nutrient solution which comprises
30 glucose, DL-pantoic acid and β -alanine, as shown in EP-A 0 493 060. EP-A 0 493 060 furthermore shows that in the case of E. coli, the formation of D-pantothenic acid is improved by amplification of pantothenic acid biosynthesis

genes from E. coli which are contained on the plasmids pFV3 and pFV5 in a nutrient solution comprising glucose, DL-pantoic acid and β -alanine.

EP-A 0 590 857 and US Patent 5,518,906 describe mutants
5 derived from E. coli strain IFO3547, such as FV5714, FV525, FV814, FV521, FV221, FV6051 and FV5069, which carry resistances to various antimetabolites, such as salicylic acid, α -ketobutyric acid, β -hydroxyaspartic acid, O-methylthreonine and α -ketoisovaleric acid. They produce
10 pantoic acid in a nutrient solution comprising glucose, and D-pantothenic acid in a nutrient solution comprising glucose and β -alanine. It is furthermore shown in EP-A 0 590 857 and US Patent 5,518,906 that after amplification of the pantothenic acid biosynthesis genes contained on the
15 plasmid pFV31, in the abovementioned strains the production of D-pantoic acid in nutrient solutions comprising glucose and the production of D-pantothenic acid in a nutrient solution comprising glucose and β -alanine is improved.

Processes for the preparation of D-pantothenic acid with
20 the aid of Corynebacterium glutamicum (C. glutamicum) are known only in some instances in the literature. Sahm and Eggeling (Applied and Environmental Microbiology 65(5), 1973-1979 (1999)) thus report on the influence of over-expression of the panB and panC genes and Dusch et al.
25 (Applied and Environmental Microbiology 65(4), 1530-1539 (1999)) report on the influence of the panD gene on the formation of D-pantothenic acid.

Object of the invention

The inventors had the object of providing new principles
30 for improved processes for the fermentative preparation of pantothenic acid with coryneform bacteria.

Description of the invention

When D-pantothenic acid or pantothenic acid or pantothenate are mentioned in the following text, this means not only the free acids but also the salts of D-pantothenic acid, such as e.g. the calcium, sodium, ammonium or potassium salt.

The invention provides a process for the fermentative preparation of D-pantothenic acid using coryneform bacteria in which the nucleotide sequence which codes for the enzyme pyruvate oxidase (EC 1.2.2.2) (poxB gene) is attenuated.

This invention also provides a process for the fermentative preparation of D-pantothenic acid, in which the following steps are carried out:

- a) fermentation of D-pantothenic acid-producing coryneform bacteria in which at least the nucleotide sequence which codes for pyruvate oxidase (EC 1.2.2.2) (poxB) is attenuated, in particular eliminated;
- b) concentration of the D-pantothenic acid in the medium or in the cells of the bacteria; and
- c) isolation of the D-pantothenic acid produced.

The strains employed optionally already produce D-pantothenic acid before attenuation of the poxB gene.

Preferred embodiments are to be found in the claims.

The term "attenuation" in this connection describes the reduction or elimination of the intracellular activity of one or more enzymes (proteins) in a microorganism which are coded by the corresponding DNA, for example by using a weak promoter or using a gene or allele which codes for a corresponding enzyme (protein) with a low activity or inactivates the corresponding gene or enzyme (protein), and optionally combining these measures.

The microorganisms which the present invention provides can produce D-pantothenic acid from glucose, sucrose, lactose, fructose, maltose, molasses, starch, cellulose or from glycerol and ethanol. They are representatives of

5 coryneform bacteria, in particular of the genus *Corynebacterium*. Of the genus *Corynebacterium*, there may be mentioned in particular the species *Corynebacterium glutamicum*, which is known among experts for its ability to produce L-amino acids.

10 Suitable strains of the genus *Corynebacterium*, in particular of the species *Corynebacterium glutamicum*, are, for example, the known wild-type strains

Corynebacterium glutamicum ATCC13032
Corynebacterium acetoglutamicum ATCC15806
15 *Corynebacterium acetoacidophilum* ATCC13870
Corynebacterium melassecola ATCC17965
Corynebacterium thermoaminogenes FERM BP-1539
Brevibacterium flavum ATCC14067
Brevibacterium lactofermentum ATCC13869 and
20 *Brevibacterium divaricatum* ATCC14020

and D-pantothenic acid-producing mutants prepared therefrom, such as, for example

Corynebacterium glutamicum ATCC13032 Δ ilvA/pEC7panBC
Corynebacterium glutamicum ATCC13032/pND-D2.

25 It has been found that coryneform bacteria produce pantothenic acid in an improved manner after attenuation of the *poxB* gene, which codes for pyruvate oxidase (EC 1.2.2.2).

"Isolated" means separated out of its natural environment.

30 "Polynucleotide" in general relates to polyribonucleotides and polydeoxyribonucleotides, it being possible for these to be non-modified RNA or DNA or modified RNA or DNA.

The nucleotide sequence of the poxB gene is shown in SEQ ID No. 1 and the resulting amino acid sequence of the enzyme protein is shown in SEQ ID No. 2.

5 The poxB gene described in SEQ ID No. 1 can be used according to the invention. Alleles of the poxB gene which result from the degeneracy of the genetic code or due to "sense mutations" of neutral function can furthermore be used.

10 A new nucleotide sequence, shown in SEQ ID No. 6, which lies upstream of the nucleotide sequence of the poxB gene region shown in SEQ ID No. 1 has been found. A new nucleotide sequence, shown in SEQ ID No. 7, which lies downstream of the nucleotide sequence of the poxB gene region shown in SEQ ID No. 1 has furthermore been found.
15 The sequence of the poxB gene region shown in SEQ ID No. 4 has been obtained in this manner.

It has been found that these polynucleotides shown in SEQ ID No. 6 and 7 are useful in the production of mutants with an attenuated, in particular eliminated, poxB gene.

20 It has also been found that coryneform bacteria produce pantothenic acid in an improved manner after attenuation of the poxB gene.

To achieve an attenuation, either the expression of the poxB gene or the catalytic properties of the enzyme protein
25 can be reduced or eliminated. The two measures can optionally be combined.

The decrease in gene expression can take place by suitable culturing or by genetic modification ("mutation") of the signal structures of gene expression. Signal structures of
30 gene expression are, for example, repressor genes, activator genes, operators, promoters, attenuators, ribosome binding sites, the start codon and terminators. The expert can find information on this e.g. in the patent

application WO 96/15246, in Boyd and Murphy (Journal of Bacteriology 170: 5949 (1988)), in Voskuil and Chambliss (Nucleic Acids Research 26: 3548 (1998)), in Jensen and Hammer (Biotechnology and Bioengineering 58: 191 (1998)),
5 in Patek et al. (Microbiology 142: 1297 (1996)) and in known textbooks of genetics and molecular biology, such as e.g. the textbook by Knippers ("Molekulare Genetik", 6th edition, Georg Thieme Verlag, Stuttgart, Germany, 1995) or that by Winnacker ("Gene und Klone", VCH
10 Verlagsgesellschaft, Weinheim, Germany, 1990).

Mutations which lead to a change or reduction in the catalytic properties of enzyme proteins are known from the prior art. Examples which may be mentioned are the works of Qiu and Goodman (Journal of Biological Chemistry 272: 8611-
15 8617 (1997)), Sugimoto et al. (Bioscience Biotechnology and Biochemistry 61: 1760-1762 (1997)) and Möckel ("Die Threonindehydratase aus Corynebacterium glutamicum: Aufhebung der allosterischen Regulation und Struktur des Enzyms", Reports from the Jülich Research Centre, Jül-2906,
20 ISSN09442952, Jülich, Germany, 1994). Summarizing descriptions can be found in known textbooks of genetics and molecular biology, such as e.g. that by Hagemann ("Allgemeine Genetik", Gustav Fischer Verlag, Stuttgart, 1986).

25 Possible mutations are transitions, transversions, insertions and deletions. Depending on the effect of the amino acid exchange on the enzyme activity, "missense mutations" or "nonsense mutations" are referred to. Insertions or deletions of at least one base pair (bp) in a
30 gene lead to "frame shift mutations", which lead to incorrect amino acids being incorporated or translation being interrupted prematurely. Deletions of several codons typically lead to a complete loss of the enzyme activity. Instructions on generation of such mutations are prior art
35 and can be found in known textbooks of genetics and

molecular biology, such as e.g. the textbook by Knippers ("Molekulare Genetik", 6th edition, Georg Thieme Verlag, Stuttgart, Germany, 1995), that by Winnacker ("Gene und Klone", VCH Verlagsgesellschaft, Weinheim, Germany, 1990) or that by Hagemann ("Allgemeine Genetik", Gustav Fischer Verlag, Stuttgart, 1986).

An example of a plasmid with the aid of which an insertion mutagenesis of the *poxB* gene can be carried out is pCR2.1poxBint (figure 1).

10 Plasmid pCR2.1poxBint comprises the plasmid pCR2.1-TOPO described by Mead et al. (Bio/Technology 9:657-663 (1991)), into which an internal fragment of the *poxB* gene, shown in SEQ-ID No. 3 has been incorporated. After transformation and homologous recombination in the chromosomal *poxB* gene
15 (insertion), this plasmid leads to a total loss of the enzyme function.

Another example of a mutated *poxB* gene is the Δ poxB allele contained in the plasmid pCRB1-poxBdel (figure 2). The Δ poxB allele contains only the 5' and the 3' flank of the
20 *poxB* gene. The 1737 bp long section of the coding region is missing (deletion). The nucleotide sequence of the Δ poxB allele and of the 5' and 3' flank is shown in SEQ ID No. 12. This Δ poxB allele can be incorporated into coryneform bacteria by integration mutagenesis. The abovementioned
25 plasmid pCRB1-poxBdel is used for this, or the Δ poxB allele is transferred to the plasmid pK18mobsacB and the plasmid of the type pK18mobsacBpoxBdel thereby formed is used. After transfer by conjugation or transformation and homologous recombination by means of a first "cross-over"
30 event which effects integration and a second "cross-over" event which effects excision in the *poxB* gene, the incorporation of the Δ poxB allele is achieved and a total loss of the enzyme function in the particular strain is achieved. The invention provides the Δ poxB allele
35 characterized by SEQ ID No. 12.

Further instructions and explanations on insertion mutagenesis or integration mutagenesis and gene replacement are to be found, for example, in Schwarzer and Pühler (Bio/Technology 9,84-87 (1991)), Peters-Wendisch et al. (Microbiology 144, 915-927 (1998)) or Fitzpatrick et al. (Applied Microbiology and Biotechnology 42, 575-580 (1994)).

It may furthermore be advantageous for the production of pantothenic acid, in addition to the attenuation of the gene which codes for pyruvate oxidase, for one or more of the genes chosen from the group consisting of

- the panB gene which codes for ketopantoate hydroxymethyl transferase (Sahm et al., Applied and Environmental Microbiology, 65, 1973-1979 (1999)),
- 15 • the panC gene which codes for pantothenate synthetase (Sahm et al., Applied and Environmental Microbiology, 65, 1973-1979 (1999)),
- the ilvC gene which codes for acetohydroxy-acid isomeroreductase (EMBL gene library: Accession No. L09232), and
- 20 • the ilvD gene which codes for dihydroxy-acid dehydratase (EP-A-1006189);

to be enhanced, in particular over-expressed.

The term "enhancement" in this connection describes the increase in the intracellular activity of one or more enzymes in a microorganism which are coded by the corresponding DNA, for example by increasing the number of copies of the gene or genes, using a potent promoter or using a gene which codes for a corresponding enzyme having a high activity, and optionally combining these measures.

It may furthermore be advantageous for the production of pantothenic acid, in addition to the attenuation of the gene which codes for pyruvate oxidase, for the pck gene which codes for phosphoenol pyruvate carboxykinase (PEP carboxykinase) (DE: 19950409.1, DSM 13047) to be attenuated.

Finally, in addition to attenuation of pyruvate oxidase, it may be advantageous for the production of pantothenic acid to eliminate undesirable side reactions (Nakayama: "Breeding of Amino Acid Producing Micro-organisms", in: Overproduction of Microbial Products, Krumphanzl, Sikyta, Vanek (eds.), Academic Press, London, UK, 1982) which reduce the production of pantothenic acid.

The microorganisms prepared according to the invention can be cultured continuously or discontinuously in the batch process (batch culture) or in the fed batch (feed process) or repeated fed batch process (repetitive feed process) for the purpose of pantothenic acid production. A summary of known culture methods are described in the textbook by Chmiel (Bioprozesstechnik 1. Einführung in die Bioverfahrenstechnik (Gustav Fischer Verlag, Stuttgart, 1991)) or in the textbook by Storhas (Bioreaktoren und periphere Einrichtungen (Vieweg Verlag, Braunschweig/Wiesbaden, 1994)).

The culture medium to be used must meet the requirements of the particular microorganisms in a suitable manner. Descriptions of culture media for more various microorganisms are contained in the handbook "Manual of Methods for General Bacteriology" of the American Society for Bacteriology (Washington D.C., USA, 1981).

Sugars and carbohydrates, such as e.g. glucose, sucrose, lactose, fructose, maltose, molasses, starch and cellulose, oils and fats, such as e.g. soya oil, sunflower oil, groundnut oil and coconut fat, fatty acids, such as e.g.

palmitic acid, stearic acid and linoleic acid, alcohols, such as e.g. glycerol and ethanol, and organic acids, such as e.g. acetic acid, can be used as the source of carbon. These substances can be used individually or as a mixture.

- 5 Organic nitrogen-containing compounds, such as peptones, yeast extract, meat extract, malt extract, corn steep liquor, soya bean flour and urea, or inorganic compounds, such as ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate and ammonium nitrate, can be
10 used as the source of nitrogen. The sources of nitrogen can be used individually or as a mixture.

- Potassium dihydrogen phosphate or dipotassium hydrogen phosphate or the corresponding sodium-containing salts can be used as the source of phosphorus. The culture medium
15 must furthermore comprise salts of metals, such as e.g. magnesium sulfate or iron sulfate, which are necessary for growth. Finally, essential growth substances, such as amino acids and vitamins, can be employed in addition to the abovementioned substances. Precursors of pantothenic acid,
20 such as aspartate, β -alanine, ketoisovalerate, ketopantoic acid or pantoic acid, and optionally salts thereof, can moreover be added to the culture medium to additionally increase the pantothenic acid production. The starting substances mentioned can be added to the culture in the
25 form of a single batch, or can be fed in during the culture in a suitable manner.

- Basic compounds, such as sodium hydroxide, potassium hydroxide, ammonia, or acid compounds, such as phosphoric acid or sulfuric acid, can be employed in a suitable manner
30 to control the pH of the culture. Antifoams, such as e.g. fatty acid polyglycol esters, can be employed to control the development of foam. Suitable substances having a selective action, e.g. antibiotics, can be added to the medium to maintain the stability of plasmids. To maintain
35 aerobic conditions, oxygen or oxygen-containing gas

mixtures, such as e.g. air, are introduced into the culture. The temperature of the culture is usually 20°C to 45°C, and preferably 25°C to 40°C. Culturing is continued until a maximum of pantothenic acid has formed. This target
5 is usually reached within 10 hours to 160 hours.

The concentration of pantothenic acid formed can be determined with known chemical (Velisek; Chromatographic Science 60, 515-560 (1992)) or microbiological methods, such as e.g. the *Lactobacillus plantarum* test (DIFCO
10 MANUAL, 10th Edition, p. 1100-1102; Michigan, USA).

The following microorganism was deposited on 19th October 1999 as a pure culture at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ = German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany)
15 in accordance with the Budapest Treaty:

- *Escherichia coli* strain DH5 α /pCR2.1poxBint as DSM 13114.

The present invention is explained in more detail in the following with the aid of embodiment examples.

Example 1

20 Preparation of a genomic cosmid gene library from *Corynebacterium glutamicum* ATCC 13032

Chromosomal DNA from *C. glutamicum* ATCC 13032 was isolated as described by Tauch et al. (1995, Plasmid 33:168-179) and partly cleaved with the restriction enzyme *Sau3AI* (Amersham
25 Pharmacia, Freiburg, Germany, Product Description *Sau3AI*, Code no. 27-0913-02). The DNA fragments were dephosphorylated with shrimp alkaline phosphatase (Roche Molecular Biochemicals, Mannheim, Germany, Product Description SAP, Code no. 1758250). The DNA of the cosmid
30 vector SuperCos1 (Wahl et al. (1987) Proceedings of the National Academy of Sciences USA 84:2160-2164), obtained from Stratagene (La Jolla, USA, Product Description

SuperCos1 Cosmid Vector Kit, Code no. 251301) was cleaved with the restriction enzyme XbaI (Amersham Pharmacia, Freiburg, Germany, Product Description XbaI, Code no. 27-0948-02) and likewise dephosphorylated with shrimp alkaline
5 phosphatase.

The cosmid DNA was then cleaved with the restriction enzyme BamHI (Amersham Pharmacia, Freiburg, Germany, Product Description BamHI, Code no. 27-0868-04). The cosmid DNA treated in this manner was mixed with the treated ATCC13032
10 DNA and the batch was treated with T4 DNA ligase (Amersham Pharmacia, Freiburg, Germany, Product Description T4-DNA-Ligase, Code no.27-0870-04). The ligation mixture was then packed in phages with the aid of Gigapack II XL Packing Extract (Stratagene, La Jolla, USA, Product Description
15 Gigapack II XL Packing Extract, Code no. 200217). For infection of the E. coli strain NM554 (Raleigh et al. 1988, Nucleic Acid Res. 16:1563-1575) the cells were taken up in 10 mM MgSO₄ and mixed with an aliquot of the phage suspension. The infection and titering of the cosmid
20 library were carried out as described by Sambrook et al. (1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor), the cells being plated out on LB agar (Lennox, 1955, Virology, 1:190) with 100 µg/ml ampicillin. After incubation overnight at 37°C, recombinant individual clones
25 were selected.

Example 2

Isolation and sequencing of the poxB gene

The cosmid DNA of an individual colony was isolated with the Qiaprep Spin Miniprep Kit (Product No. 27106, Qiagen,
30 Hilden, Germany) in accordance with the manufacturer's instructions and partly cleaved with the restriction enzyme Sau3AI (Amersham Pharmacia, Freiburg, Germany, Product Description Sau3AI, Product No. 27-0913-02). The DNA fragments were dephosphorylated with shrimp alkaline

phosphatase (Roche Molecular Biochemicals, Mannheim, Germany, Product Description SAP, Product No. 1758250). After separation by gel electrophoresis, the cosmid fragments in the size range of 1500 to 2000 bp were
5 isolated with the QiaExII Gel Extraction Kit (Product No. 20021, Qiagen, Hilden, Germany).

The DNA of the sequencing vector pZero-1, obtained from Invitrogen (Groningen, The Netherlands, Product Description Zero Background Cloning Kit, Product No. K2500-01) was
10 cleaved with the restriction enzyme BamHI (Amersham Pharmacia, Freiburg, Germany, Product Description BamHI, Product No. 27-0868-04). The ligation of the cosmid fragments in the sequencing vector pZero-1 was carried out as described by Sambrook et al. (1989, Molecular Cloning: A
15 Laboratory Manual, Cold Spring Harbor), the DNA mixture being incubated overnight with T4 ligase (Pharmacia Biotech, Freiburg, Germany). This ligation mixture was then electroporated (Tauch et al., 1994, FEMS Microbiol Letters, 123:343-7) into the E. coli strain DH5 α MCR (Grant, 1990,
20 Proceedings of the National Academy of Sciences, U.S.A., 87:4645-4649) and plated out on LB agar (Lennox, 1955, Virology, 1:190) with 50 μ g/ml zeocin.

The plasmid preparation of the recombinant clones was carried out with the Biorobot 9600 (Product No. 900200,
25 Qiagen, Hilden, Germany). The sequencing was carried out by the dideoxy chain termination method of Sanger et al. (1977, Proceedings of the National Academies of Sciences, U.S.A., 74:5463-5467) with modifications according to Zimmermann et al. (1990, Nucleic Acids Research, 18:1067).
30 The "RR dRhodamin Terminator Cycle Sequencing Kit" from PE Applied Biosystems (Product No. 403044, Weiterstadt, Germany) was used. The separation by gel electrophoresis and analysis of the sequencing reaction were carried out in a "Rotiphoresis NF Acrylamide/Bisacrylamide" Gel (29:1)
35 (Product No. A124.1, Roth, Karlsruhe, Germany) with the

"ABI Prism 377" sequencer from PE Applied Biosystems (Weiterstadt, Germany).

The raw sequence data obtained were then processed using the Staden program package (1986, Nucleic Acids Research, 14:217-231) version 97-0. The individual sequences of the pZerol derivatives were assembled to a continuous contig. The computer-assisted coding region analysis were prepared with the XNIP program (Staden, 1986, Nucleic Acids Research, 14:217-231).

The resulting nucleotide sequence is shown in SEQ ID No. 1. Analysis of the nucleotide sequence showed an open reading frame of 1737 base pairs, which was called the poxB gene. The poxB gene codes for a polypeptide of 579 amino acids shown in SEQ ID No. 2.

Example 3

Preparation of the integration vector pCR2.1poxBint for mutagenesis of the poxB gene

From the strain ATCC 13032, chromosomal DNA was isolated by the method of Eikmanns et al. (Microbiology 140: 1817-1828 (1994)). On the basis of the sequence of the poxB gene known for *C. glutamicum* from example 2, the following oligonucleotides were chosen for the polymerase chain reaction:

poxBint1:

5' TGC GAG ATG GTG AAT GGT GG 3'

poxBint2:

5' GCA TGA GGC AAC GCA TTA GC 3'

The primers shown were synthesized by MWG Biotech (Ebersberg, Germany) and the PCR reaction was carried out by the standard PCR method of Innis et al. (PCR protocols. A Guide to Methods and Applications, 1990, Academic Press) with Pwo-Polymerase from Boehringer. With the aid of the

polymerase chain reaction, a DNA fragment approx. 0.9 kb in size was isolated, this carrying an internal fragment of the poxB gene and being shown in SEQ ID No. 3.

The amplified DNA fragment was ligated with the TOPO TA Cloning Kit from Invitrogen Corporation (Carlsbad, CA, USA; Catalogue Number K4500-01) in the vector pCR2.1-TOPO (Mead et al. (1991) Bio/Technology 9:657-663). The E. coli strain Top10F' (Grant et al. (1990) Proceedings of the National Academy of Sciences, USA, 87:4645-4649) was then electroporated. Selection for plasmid-carrying cells was made by plating out the transformation batch on LB agar (Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989), which had been supplemented with 50 mg/l kanamycin. Plasmid DNA was isolated from a transformant with the aid of the QIAprep Spin Miniprep Kit from Qiagen and checked by restriction with the restriction enzyme EcoRI and subsequent agarose gel electrophoresis (0.8%). The plasmid was called pCR2.1poxBint (figure 1).

20 Example 4

Preparation of an exchange vector for deletion mutagenesis of the poxB gene

4.1 Determination of the nucleotide sequence of the flanks of the poxB gene

25 In further sequencing steps, the nucleotide sequence of the poxB gene region shown in SEQ ID No. 1 was extended upstream and downstream by in each case approx. 500 to 600 bp. The method described in example 2 was used for this. The extended nucleotide sequence of the poxB gene region shown in SEQ ID No. 4 was obtained in this manner. 30 The new upstream of the poxB gene region shown in SEQ ID No. 1 is shown in SEQ ID No. 6. The new downstream of the

poxB gene region shown in SEQ ID No. 1 is shown in SEQ ID No. 7.

4.2 Construction of a Δ poxB allele

The method of geneSOEing-PCR described by Horton (Molecular Microbiology 3:93-99 (1995)) was used for construction of the Δ poxB allele. The primer pairs shown in table 1 (see also SEQ ID No. 8 to 11) were constructed for this. By means of a PCR, the 5' region before the poxB gene was amplified with primer pair 1 and the 3' region after the poxB gene was amplified with primer pair 2. A further PCR was then carried out with the two amplification products and the primers pox-del1 and pox-del4, as a result of which the two amplification products were joined by means of geneSOEing. The deletion fragment or Δ poxB allele obtained in this way contains the flanking sequences of the poxB gene. The nucleotide sequence of the Δ poxB allele is shown in SEQ ID No. 12.

Table 1

Primer	5'-Sequence-3'	Primer pair
pox-del1	ATGAGGAACATCCGGCGGTG	1
pox-del2	GAGAACAGCAGGAGTATCAATCATCACTGAACT CCTCAACGTTATGGC	
pox-del3	TGATGATTGATACACCTGCTGTTCTC	2
pox-del4	TCATTGCCACCTGCTTCTCA	

4.3 Construction of an exchange vector

The DNA fragment obtained in this way was ligated with the Zero Blunt TOPO PCR Cloning Kit from Invitrogen Corporation (Carlsbad, CA, USA; Catalogue Number K2800-20) in the

vector pCR-Blunt II-TOPO vector (Shuman et al., (1994) Journal of Biological Chemistry 269:32678-32684; Bernard et al., (1983) Journal of Molecular Biology 234:534-541). The E. coli strain Top10 (Grant et al. (1990) Proceedings of the National Academy of Sciences, USA 87:4645-4649) was then transformed with the ligation batch. Selection for plasmid-carrying cells was made by plating out the transformation batch on LB agar (Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.), which had been supplemented with 50 mg/l kanamycin. Plasmid DNA was isolated from a transformant with the aid of the QIAprep Spin Miniprep Kit from Qiagen and checked by restriction with the restriction enzyme EcoRI and subsequent agarose gel electrophoresis (0.8%). The plasmid was called pCRB1-poxBdel (figure 2).

The insert carrying the Δ poxB allele was excised from this plasmid by means of EcoRI, isolated from the gel and ligated in the non-replicative integration vector pK18mobsacB, which was also cleaved with EcoRI (Schafer et al., Gene 145, 69-73 (1994)). The clonings were carried out in E. coli DH5 α mcr (Grant et al., (1990) Proceedings of the National Academy of Sciences, USA, 87: 4645-4649) as the host. The resulting plasmid was called pK18mobsacB-poxBdel.

25 Example 5

Mutagenesis of the poxB gene in the strain FERM BP-1763

The L-valine-producing strain *Brevibacterium lactofermentum* FERM BP-1763 is described in the patent specification US-A-5,188,948.

30 For deletion of the poxB gene, the integration plasmid pK18mobsacB-poxBdel was electroporated in the strain FERM BP-1763. After selection for kanamycin (25 μ g/ml), individual clones in which the inactivation vector was

present integrated in the genome were obtained. To allow excision of the vector, individual colonies were incubated in 50 ml liquid LB medium without antibiotics for 24 hours at 30°C and 130 rpm and then smeared on to sucrose-
5 containing agar plates (LB with 15 g/l agar and 10% sucrose). Clones which had lost the vector content again by a second recombination event were obtained by this selection (Jäger et al. 1992, Journal of Bacteriology 174:5462-5465). To identify those clones which carried the
10 ΔpoxB allele, a polymerase chain reaction was carried out with the primers pox-del1 and pox-del4 (table 1 and SEQ ID No. 8 and 11). These primers amplify on the whole DNA of the starting strain FERM BP-1763 a fragment approx. 3150 bp in size, while on the DNA of poxB deletion mutants the
15 primers amplified a shortened fragment 1422 bp in size. A deletion mutant identified in this way is consequently lacking a region of the poxB gene 1.7 kb in size.

A strain produced and tested in this manner was called FERM BP-1763ΔpoxB and employed for further studies.

20 Example 6

Preparation of pantothenic acid

6.1 Production of the strains

The plasmid pND-DBC2, which carries the panB, panC and panD genes of *Corynebacterium glutamicum*, is known from EP-A-
25 1006192. The plasmid is deposited in the form of the strain ATCC13032/pND-DBC2 as DSM 12437 at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ = German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) in accordance with the Budapest
30 Treaty.

The pantothenic acid-producing strains FERM BP-1763/pND-DBC2 and FERM BP-1763ΔpoxB were formed by transformation of

the strains FERM BP-1763 and FERM BP-1763 Δ poxB with the plasmid pND-DBC2.

6.2 Preparation of pantothenic acid

In each case a sample of the strains *Brevibacterium*
5 lactofermentum FERM BP-1763/pND-DBC2 and FERM BP-1763 Δ poxB/pND-DBC2 was smeared on to HHK agar.

HHK agar comprises brain-heart agar, which was obtained from Merck KgaA (Darmstadt, Germany) and supplemented with kanamycin. The composition of the HHK agar is shown in
10 table 2.

This agar plate culture was incubated for 17 hours at 37°C and then kept in a refrigerator at +4°C. Selected individual colonies were then propagated further on the same medium. Cell material of a clone was removed from the
15 HHK agar with an inoculating loop and transferred to 100 mL HHK broth contained in a shaking flask of 1000 mL total volume.

HHK broth comprises brain-heart medium, which was obtained from Merck KgaA (Darmstadt, Germany) and supplemented with
20 glucose and kanamycin. The composition of the HHK broth is shown in table 3.

Table 2

HHK agar

Substance	Amount per litre
Brain-heart agar	52.0 g
Kanamycin	25 mg

Table 3

HHK broth

Substance	Amount per litre
Brain-heart medium	37.0 g
Kanamycin	25 mg
Glucose	20.0 g

- The batches were incubated at 30°C and 150 rpm for
- 5 22 hours. After the end of the culturing, an optical density of in each case approx. 6 was measured in a photometer at a wavelength of 660 nm (OD 660). This culture of the strain was used to inoculate the production fermenter.
- 10 Medium SK-71 shown in table 4 was used for the fermentation. All the components of the SK-71 medium were initially introduced into the fermenter directly according to the working concentration and sterilized in situ.

Table 4

Medium SK-71

Compound	Amount per litre
Glucose hydrate	110.0000g
Corn steep liquor (CSL)	5.0000g
β -Alanine	5.0000g
Nicotinic acid	0.0050g
l-Isoleucine	0.1500g
Homoserine	0.1500g
Ammonium sulfate	25.0000g
K dihydrogen phosphate	0.1000g
Mg sulfate 7H ₂ O	1.0000g
Fe sulfate 7H ₂ O	0.0100g
Mn sulfate H ₂ O	0.0050g
CaCl ₂ * 2H ₂ O	0.0100g
Thiamine HCl	0.0002g
D(+)Biotin	0.0003g
Structol	0.60g

10 l stirred reactors from B.Braun (BBI, Germany,
5 Melsungen, Biostat E/ED model) were used as the fermenters.

For inoculation of 1950 g of the fermentation medium SK-71,
in each case 100 mL of the shaking flask precultures in HHK
broth described above were employed.

10 The batch was cultured over the entire fermentation time at
a temperature of 30°C, a volume-specific aeration of
0.75 vvm, stirring, dependent on the oxygen consumption, of

800 - 1700 rpm and a pH of 7.0 and an oxygen partial pressure of 20% of the atmospheric saturation. The culture was cultured for a total of approx. 49 hours under the abovementioned conditions until an OD₆₆₀ of approx. 2.6 was reached. An aqueous ammonia solution (25 % w/v) was used as the correcting agent for regulating the pH.

The optical density (OD) was then determined with a digital photometer of the type LPlW from Dr. Bruno Lange GmbH (Berlin, Germany) at a measurement wavelength of 660 nm and the concentration of D- pantothenic acid formed was determined by means of HPLC (Hypersil APS 2 5 µm, 250x5 mm, RI detection).

A D-pantothenic acid concentration of approx. 0.20 g/l was measured in the end sample (approx. 49 hours) of the fermentation culture of the strain FERM BP-1763/pND-DBC2. The pantothenic acid concentration in the corresponding sample of the strain FERM BP-1763ΔpoxB/pND-DBC2 was approx. 0.23 g/l.

The following figures are attached:

- Figure 1: Map of the plasmid pCR2.1poxBint
- Figure 2: Map of the plasmid pCRB1-poxBdel

The base pair numbers stated are approx. values obtained in the context of reproducibility.

The abbreviations and designations used have the following meaning:

Figure 1:

AmpR	Ampicillin resistance gene
ColE1 ori	Replication origin ColE1
f1 ori	Replication origin of phage f1
KmR	Kanamycin resistance gene

lacZ	Residues of the lacZ α gene fragment
poxBint	Internal fragment of the poxB gene

Figure 2:

'lacZa	3' end of the lacZ α gene fragment
3'-Region	3' flank of the poxB gene
5'-Region	5' flank of the poxB gene
ccdB	ccdB gene
Km	Kanamycin resistance gene
lacZa'	5' end of the lacZ α gene fragment
plac.	Promoter of the lac operon
pMB1	Replication origin of the plasmid pMB1
Zeocin	Zeocin resistance gene

The following abbreviations have moreover been used:

BamHI:	Cleavage site of the restriction enzyme BamHI
ClaI	Cleavage site of the restriction enzyme ClaI
EcoRI:	Cleavage site of the restriction enzyme EcoRI
HindIII:	Cleavage site of the restriction enzyme HindIII
SalI:	Cleavage site of the restriction enzyme SalI

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Deletion of the coding region of the poxB gene

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Patent claims

1. A process for the fermentative preparation of D-pantothenic acid, wherein the following steps are carried out:
 - 5 a) fermentation of D-pantothenic acid-producing coryneform bacteria in which at least the nucleotide sequence which codes for pyruvate oxidase (EC 1.2.2.2) (poxB) is attenuated, in particular eliminated;
 - 10 b) concentration of the D-pantothenic acid in the medium or in the cells of the bacteria; and
 - c) isolation of the D-pantothenic acid produced.
2. The process as claimed in claim 1, wherein the process of insertion, in particular by means of the vector
15 pCR2.1poxBint, shown in figure 1 and deposited in E.coli as DSM 13114, is used to achieve the attenuation.
3. The process as claimed in claim 1, wherein the process of deletion, in particular by means of the vector
20 pCRB1-poxBdel, shown in figure 2, is used to achieve the attenuation.
4. The process as claimed in claim 1, wherein coryneform bacteria in which further genes of the biosynthesis pathway of D-pantothenic acid are additionally enhanced
25 are employed.
5. The process as claimed in claim 1, wherein coryneform bacteria in which the metabolic pathways which reduce the formation of D-pantothenic acid are at least partly eliminated are employed.
- 30 6. The process as claimed in claim 3, wherein coryneform bacteria in which at the same time the panB gene which

codes for ketopantoate hydroxymethyl transferase is enhanced are employed.

- 5 7. The process as claimed in claim 3, wherein coryneform bacteria in which at the same time the panC gene which codes for pantothenate is enhanced are employed.
8. The process as claimed in claim 3, wherein coryneform bacteria in which at the same time the ilvC gene which codes for acetohydroxy-acid isomeroreductase is enhanced are employed.
- 10 9. The process as claimed in claim 3, wherein coryneform bacteria in which at the same time the ilvD gene which codes for dihydroxy-acid dehydratase is enhanced are employed.
- 15 10. The process as claimed in claim 1 or 4, wherein coryneform bacteria in which at the same time the pck gene which codes for phosphoenol pyruvate carboxykinase is attenuated are employed.
- 20 11. The process as claimed in claims 5 to 8, wherein the genes mentioned are enhanced in coryneform bacteria which already produce D-pantothenic acid.
12. The process as claimed in claim 9, wherein coryneform bacteria which already produce D-pantothenic acid and in which the pck gene is attenuated are employed.
- 25 13. An isolated polynucleotide from coryneform bacteria which lies upstream of SEQ ID No. 1 and is shown in SEQ ID No. 6.
14. An isolated polynucleotide from coryneform bacteria which lies downstream of SEQ ID No. 1 and is shown in SEQ ID No. 7.

15. An isolated polynucleotide from coryneform bacteria, containing a deletion mutation of the poxB gene shown in SEQ ID No. 12.
 16. Coryneform bacteria which carry the deletion mutation shown in SEQ ID No. 12.
- 5

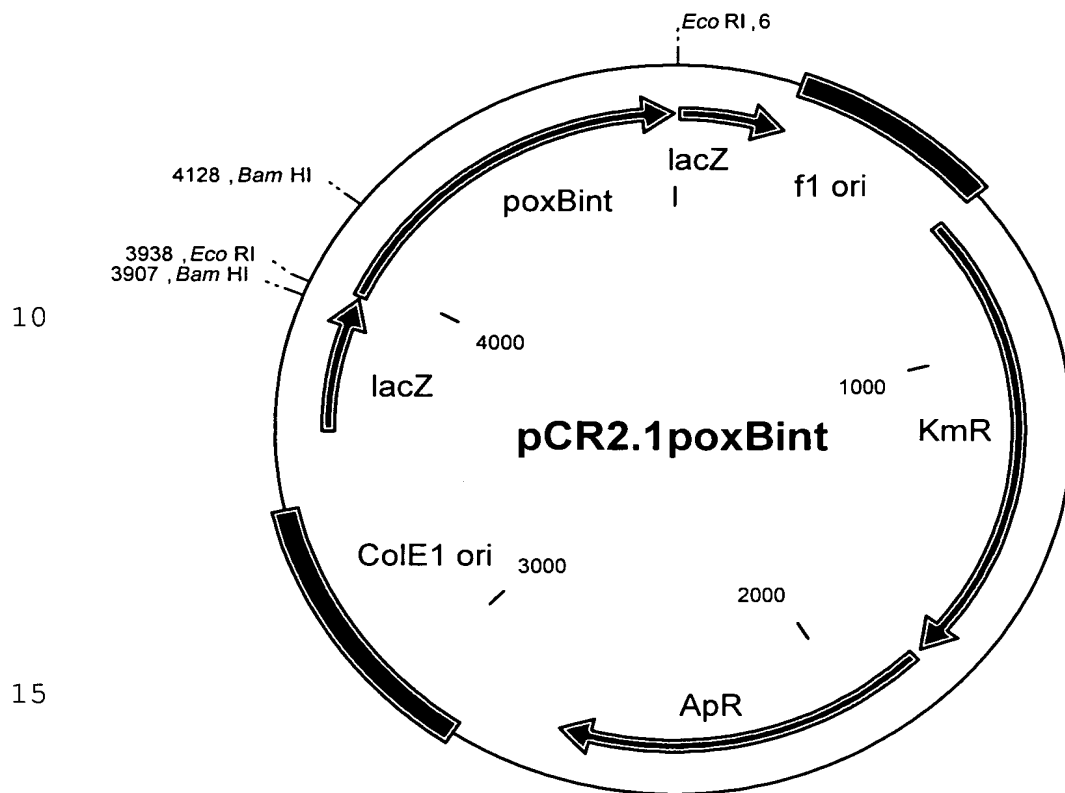
Abstract

The invention relates to a process for the preparation of D-pantothenic acid by fermentation of coryneform bacteria, in which bacteria in which the nucleotide sequence which
5 codes for pyruvate oxidase (EC 1.2.2.2) (poxB gene) is attenuated are employed, the following steps being carried out:

- 10 a) fermentation of D-pantothenic acid-producing bacteria in which at least the gene which codes for pyruvate oxidase is attenuated.
- b) concentration of the D-pantothenic acid in the medium or in the cells of the bacteria; and
- c) isolation of the D-pantothenic acid produced.

Figure 1: Map of the plasmid pCR2.1poxBint

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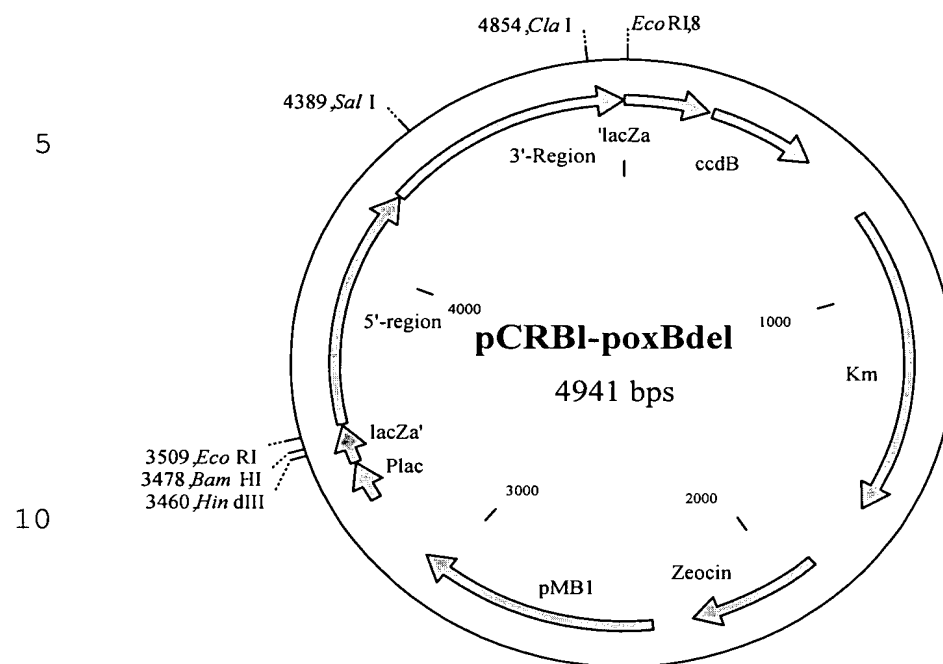


15

20

Figure 2:

3



15

20